dilated intestinal bed and not to reduced outflow through the hepatic circuit, whose resistance at this time had returned to the control value. Evidence for such increase in intestinal flow of the monkey was not seen in the present series.

Such functional differences may account for the difference in appearance of the intestines at autopsy, for the apparent hyperemia manifested in the canine mesenteric bed would appear to be related to congestion. The reason for difference in behavior of the splanchnic circulation in the dog and monkey cannot now be explained. It is now conceded that splanchnic pooling in the dog is not as significant as was formerly believed (4,5), but it has been suggested that the observed congestive intestinal changes, which involve breakdown of the mucosa and its vasculature, might release toxic substances into the circulation(6). These might be proteolytic breakdown products or metabolites; alternatively, the lesions might permit the escape of substances such as endotoxins and tyramines from the intestinal lumen. In the presence of a liver damaged by anoxia, these substances might more readily invade the systemic circulation, with deleterious consequences(7). Another possibility is that the canine intestinal changes predispose this species to loss of significant amounts of fluid and blood into the bowel, to be irretrievably lost as bloody diarrhea.

If such mechanisms prove important in contributing to the fatal consequence of shock in the dog, it does not appear likely, based on present evidence, that such are applicable to the monkey. Thus, significant differences in the etiology of irreversible shock in the primate and canine appear to be indicated.

Summary. Analysis of pressure gradients in the splanchnic bed of the monkey during a standardized hemorrhagic shock procedure has indicated a preponderant increase in intrahepatic resistance with minimal change in mesenteric (intestinal) resistance during oligemia. Following restoration of the bleeding volume, the control pressure relationship was reestablished. The marked elevation of portal venous pressure, so typical of the dog in this phase of the hemorrhagic shock procedure, was not seen in the monkey. Moreover, the intestine of this species appeared pale and ischemic at autopsy, contrasted to the congested and bloody intestine of the dog. The possible significance of the differences in behavior of splanchnic vasculature in the primate and canine species is considered in the light of the etiology of irreversible shock.

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Susceptibility of a Trachoma Agent Grown in FL Cell Cultures to Antibiotics and a Sulfa Drug.* (27706)

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In most of the reports of studies on the effect of antibiotics and other drugs on the agent of trachoma, fertilized eggs or laboratory animals have been employed as the host system(1,2,3,4,5,6,7). Recently it has become possible to grow the T'ang strain TE55 of trachoma in cell cultures(8,9,10) and to examine its susceptibility to drugs.

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The growth curve of the agent in FL cells has been reported(11,12). The period of a single growth cycle lasts 28 hours. As infection of the cells is not synchronous, it takes 72 hours for maximal infectivity to be reached in the culture. A series of characteristic morphological and biochemical changes occurs during the intracellular development of the agent, which is only infective at the elementary body stage. The elementary bodies stain red with Giemsa and show a green fluorescence when stained with acridin orange and examined in the fluorescence microscope, due to the presence of large amounts of DNA. Inside the cytoplasm of susceptible cells the elementary bodies seem to increase in size. These larger bodies, which are non-infective, stain blue with Giemsa and red with acridin orange, due to the presence of large amounts of RNA. The bodies further increase in size and within them smaller elements appear, until the elementary body stage is reached again, and with it infectivity reappears.

The effect of a number of antibiotics and of a sulfa drug on the agent during its different stages of development in cell culture is described here.

Materials and methods. Cell cultures. All experiments were carried out in FL cell cultures(13) grown in ordinary test tubes or Leighton tubes in 1 ml of LY medium composed of 80% Earle's solution containing 0.45% glucose, 0.1% yeast extract (Difco) and 0.5% lactalbumin hydrolysate (N.B. Co.) and 20% inactivated human serum. The medium also contained 0.001% phenol red, 200 μg/ml streptomycin and 10 units/ml nystatin. Cells were obtained from stock cultures grown in milk bottles in LY medium which contained in adition to the above mentioned antibiotics 200 units/ml penicillin. As it was found(1) that penicillin inhibits the growth of trachoma agent, FL cells were treated with versene solution and washed with LY medium without serum and penicillin, suspended in growth medium without this antibiotic and seeded to tubes. After 3-4 days the medium was changed and the cultures were infected with the T'ang strain of trachoma agent.

For microscopic examination cell cultures grown on coverslips in Leighton tubes were washed with a buffer solution (pH 7), fixed with methyl alcohol or Carnoy's solution and stained with Giemsa or acridin orange(14) respectively. Preparations stained by the latter method were examined with the fluorescence microscope.

Trachoma agent. The T'ang strain of trachoma (TE55) obtained from Dr. L. H. Collier, London, was grown in FL cells(8), and used between the 10th and the 90th passage level. Stock suspensions of the agent were prepared from FL cell cultures 3 days after infection. The cells were then scraped off the glass into the medium, after its acidity had been neutralized by addition of one drop of N/10 NaOH, and pipetted repeatedly through a finely drawn out capillary pipette to break up the large vacuoles which were filled with infective particles. The resulting suspensions contained up to 2.8×10^7 inclusion forming units per ml (see below). Cultures 3-4 days old were infected by adding to the culture medium 0.1 ml of such suspensions, diluted 1:5 - 1:8, and reincubated at 37°C for 72 hours. Under these conditions practically all cells became infected and showed the characteristic inclusions.

In experiments in which infectivity titers were determined the cultures were broken up by ultrasonic treatment. For this purpose the cells were suspended in medium and treated for 40 seconds in a MSE disintegrator with a power output of 60 watts at a frequency of 20 kc/sec. The number of infective particles was determined by titration in fresh FL monolayer cultures grown on coverslips in Leighton tubes. Three days later these cultures were fixed, Giemsa stained and the number of vacuoles in them was counted in at least 50 fields. The number of inclusion forming units was determined according to the formula of Furness *et al.*(15).

Drugs examined. The following antibiotics were examined: tetracycline (Lederle), oxytetracycline (Lederle), chlortetracycline (Lederle), chloramphenicol (Abic), erythromycin (Abbott), penicillin V (Teva), penicillin G (Rafa), streptomycin (Rafa), bacitracin (Ciba), neomycin (Ciba), nystatin (Squibb). Experiments were also carried out with the sulfa drug sulfanilamido-3phenyl-2pyrazole

TABLE I. Effect of Antibiotics and a Sulfa Drug on T'ang Strain (TE55) of Trachoma Agent in FL Cell Cultures.

Drug*	Minimal dose (μg/ml) causing Complete inhibition	Partial inhibition
Oxytetracycline	.1	.0001
Tetracycline	.1	.001
Chloramphenicol	1.0	.01
Erythromycin	1.0	.01
Chlortetracycline	10.0	1.0
Penicillin	.1 unit†	.01 unit
Orisul	100.0†	

* FL cell cultures in Leighton tubes were infected with 0.1 ml of a suspension of the T'ang strain of trachoma. 0.1 ml amounts of the antibiotics and the sulfa drug were added immediately and the cells reincubated at 37°C. After 72 hr, cultures were fixed and stained with Giemsa and acridin orange. Examinations for infectivity were carried out by transfer of sonicated cell suspensions to fresh monolayer cultures.

† At these doses trachoma agent passed through some early stages of its developmental cycle but infective particles were not observed.

(Orisul, Ciba) and with aminopterin (Lederle). Stock solutions of all drugs employed were prepared in sterile distilled water. Alkaline distilled water was used to dissolve the sulfa drug. Further dilutions of all drugs were prepared in LY medium without serum, and amounts of 0.1 ml were added to cultures immediately or at different intervals after infection. Control cultures received 0.1 ml of LY medium only.

For the inactivation of penicillin a purified standardized penicillinase preparation(16) supplied by Dr. N. Citri, Department of Bacteriology, Hebrew University, Jerusalem, was added in amounts sufficient to neutralize the drug without damaging the cultures. Solutions of para-amino-benzoic acid (PABA), folic acid and folinic acid (citrovorum factor) prepared in distilled water were used to reverse the effect of the sulfa drug.

Results. Streptomycin, neomycin and bacitracin were without effect on the agent. When doses between 100 and 1000 μg/ml were added to the medium immediately after infection the development of the agent was as in untreated control cultures. All succeeding experiments were carried out in the presence of 200 μg/ml of streptomycin. Also included in the medium were 10 units/ml of nystatin which proved to have no effect on the development of the agent.

The other antibiotics examined inhibited the growth cycle of the T'ang (TE55) strain (Table I). Doses of 0.1 μg/ml of tetracycline and oxytetracycline and of 10 μg/ml of chlortetracycline, when added to the culture medium immediately after infection of the cells, completely prevented multiplication of the agent. In the presence of these and higher concentrations of the drugs the infective particles were taken up by the cells, but only few of them underwent a slight increase in size and a change in their staining characteristics within 72 hours.

At concentrations 10-1000 times lower than the dose necessary for complete inhibition, these antibiotics still exerted a marked effect. Under these conditions the infective particles in the cytoplasm developed into small inclusions, which appeared as irregularly formed masses, and took a blue violet stain with Giemsa and appeared red after staining with acridin orange. In concentrations of the drugs approaching the lower limit of effectiveness a small number of particles of elementary body size, which stained red with Giemsa and green with acridin orange, were present in the developing inclusions. The transition from complete inhibition to the absence of any inhibition was a gradual one, covering a hundred- to thousand-fold range in the case of tetracycline and oxytetracycline, in contrast to chlortetracycline, where the range was only 10-fold.

In the preceding experiments the drugs were added to the cell cultures immediately after infection. It was of interest to determine whether the drugs inhibited the growth cycle of the agent when added at later stages. For this purpose a number of experiments were carried out with tetracycline. Microscopic examination of Giemsa-stained preparations showed that the drug even when added during the later stages of the growth cycle inhibited the development of infectivity. No infectivity could be demonstrated in cultures at 72 hours after infection to which tetracycline was added as late as 35 hours after infection. Even 52 hours after infection the

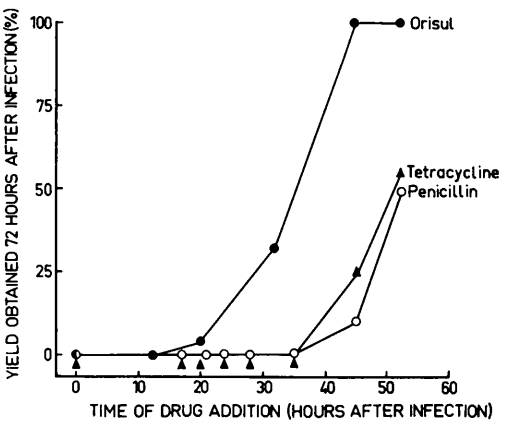

FIG. 1. Effect of penicillin, tetracycline and orisul, added at different intervals after infection, on a trachoma agent in FL cells. The cell cultures were infected with the TE55 strain of trachoma. Penicillin (100 u/ml), tetracycline (0.1 μg/ml) and orisul (10 μg/ml) were added to the culture medium at different time intervals after infection, and the cultures were reincubated for 72 hr at 37°C. The cells were treated in an ultrasonic disintegrator and the yield of infective particles was determined (see *Methods*).

drug still exerted some inhibitory effect (Fig. 1).

Chloramphenicol and erythromycin in doses of 1.0 μg/ml also completely inhibited the growth of the agent when added to the culture media immediately after infection. These drugs still showed some effect in amounts of 0.01 μg/ml. Oxytetracycline and tetracycline were the most effective drugs, followed by chloramphenicol, erythromycin and chlortetracycline (Table I).

The effect of penicillin differed from that of the antibiotics mentioned above. Even in large doses it failed to cause complete suppression of the development of the agent. At concentration of 1000-0.1 units/ml of penicillin G and V the vacuoles which appeared in the cytoplasm of the infected cells at 24-48 hours contained large irregularly formed masses staining an uneven bluish-red with Giemsa. Preparations stained with acridin orange and examined by fluorescence microscopy revealed that these structures took on a red color (RNA type). In later stages of the development green staining material (DNA type) was seen lying next to and around the masses. In the presence of 0.1 unit/ml a few particles of elementary body size also appeared in a small number of the inclusions. They became much more numerous at a concentration of 0.01 unit/ml of penicillin. At this concentration many mature vacuoles were already present. In a number of experiments trachoma infected, penicillin treated cultures after 72 hours incubation were examined for the presence of infective particles. For this purpose sufficient penicillinase was added to the culture medium to neutralize the antibiotic. No evidence of infectivity was observed when penicillin in concentrations of 0.1 or more units/ml had been added to the cultures at zero time. In the presence of 0.01 unit/ml of penicillin many infective particles developed in the inclusions, but only 50% or less of the infectivity in untreated controls was reached. Even at this low concentration, penicillin showed some obvious inhibition. The action of penicillin, when added at different intervals after infection, was also examined. Penicillin, in a concentration of 100 units/ml, completely prevented the appearance of infective particles when added as late as 28 hours. Addition of the drug 35, 45 and 52 hours after infection gave rise to 1, 10 and 50% of the maximal yield of the controls, respectively (Fig. 1). The inhibiting action of penicillin, like that of tetracycline, is obviously not limited to the early stages of the development.

The drug sulfanilamido-3phenyl-2pyrazole, like penicillin, did not completely inhibit the development of the agent even when large doses (100 μg/ml) were employed. Small vacuoles developed which contained a small number of particles staining red with acridin orange. In some vacuoles a few yellow-green colored particles were also visible. However, on transfer of such cultures to fresh monolayers infectivity could not be demonstrated. In infected cultures, treated with 10 μg/ml of the drug, the vacuoles which developed were filled with a somewhat larger number of particles staining partly red and partly yellow-green with acridin orange. At this concentration of the drug, infectivity could be demonstrated as shown by transfer to fresh cultures. Mature inclusions appeared in the presence of 1 μg/ml, although they were still

smaller in size than in untreated control cultures.

It was also of interest to determine the action of the sulfa drug when added at different intervals after infection. Complete inhibition was observed when the drug was added during the first 12 hours after infection. When added at 20 and 32 hours after infection 4.4% and 32%, respectively, of the yield reached in untreated controls, after 72 hours, were obtained. At 45 hours and later the drug was without effect (Fig. 1). The action of the sulfa drug was more time limited than that of tetracycline and penicillin.

The partial inhibition by 10 μg/ml of the sulfa drug could be completely reversed by addition of 1 μg/ml PABA, 10 μg/ml folinic acid or 100 μg/ml folic acid to the culture medium. Smaller amounts were without effect. These findings indicate the importance of the folic acid complex for development of the agent. It is in agreement with these results that aminopterin in amount of 1 μg/ml inhibited the agent. The effect could be completely reversed by 10 μg/ml of folinic acid. In the presence of aminopterin small vacuoles developed, which contained some early noninfective stages of the agent.

Discussion. Use of cell cultures instead of fertilized eggs or experimental animals as an assay system for evaluation of antitrachomatous drugs has several advantages. The effect on the agent can at every stage be determined by direct microscopic examination. The minimal inhibiting doses are 10-100 times smaller in cell cultures than those reported from experiments in which eggs or white mice (1,2,3,4,5,6,7) were employed. A partial inhibitory effect can be demonstrated in still lower doses. For preliminary screening of drugs for antitrachoma effects cell cultures are well suited, even though the doses used for treatment of patients will always be substantially above those effective in laboratory tests, as has been pointed out before(4).

The drugs streptomycin, bacitracin and neomycin, did not exert an inhibitory effect on the development of the T'ang strain of trachoma, even when concentrations as high as 1000 μg/ml were employed. The slight inhibitory effect of streptomycin, at a concentration of 64 μg/ml, on trachoma agents cultivated in chick endothelial cells reported by Gordon and Quan(17) was not observed under our conditions. The absence of any antitrachomatous activity of bacitracin and nystatin reported by these authors was confirmed. Our experiments show that neomycin can be added to the antibiotics without antitrachoma effect.

Of the 6 other antibiotics tested, tetracycline and oxytetracycline were the most effective. It was shown that tetracycline prevented further development of the agent even at late stages of its cycle. The present observations do not explain the mechanism of action of these substances.

Penicillin proved inhibitory in our experiments when used in concentrations as low as 0.1 unit/ml. Under these conditions abnormal inclusions appeared and no infective particles developed. This finding is not in agreement with those of Pollard and Tanami(18) that only partial inhibition was obtained when concentrations of 100 units/ml were employed. Penicillin resembled tetracycline also in inhibiting at the later stages of the developmental cycle.

Examination of acridin orange stained preparations revealed that in the presence of penicillin both RNA and DNA containing materials were synthesized inside the inclusion but incorporation into well defined structures was not accomplished. Observations with the electron microscope(11) have shown that the failure of the agent to complete its developmental cycle in a normal manner in the presence of penicillin is apparently due to a disturbance in formation of the membranes inside the inclusions, which are necessary for the appearance of the infective particles. It is of interest that formation of the outer membrane of the inclusion was not inhibited by penicillin, suggesting that it may be of a different composition than the membranes of the inner bodies. In bacteria penicillin was found to inhibit cell wall synthesis (19). The inhibitory effect of penicillin on formation of membranes of certain developmental stages of the trachoma agent suggests the presence of similar compounds in these structures.

The sulfa drug sulfamido-3phenyl-2pyrazole when added in doses of 100 μg/ml resembled penicillin in preventing the appearance of infective particles without completely inhibiting the developmental cycle. The drug completely inhibited the formation of infective particles when added during the first 12 hours after infection. It behaved in this respect differently from penicillin and tetracycline which also inhibited at later stages. In the presence of 10-100 times smaller doses the infectivity titer was still much lower than in untreated control cultures. The inhibiting effect could be reversed by simultaneous addition of PABA, folinic or folic acids. These findings are in complete agreement with the classical experiments of Morgan and his associates(20,21,22), who showed that the inhibiting effect of sulfadiazine on the agent of psittacosis could be similarly reversed. Pollard and Tanami(18) did not observe an inhibiting effect of sulfanilamide on the T'ang strain of trachoma in cell culture, possibly because they carried out their experiments in another cell line grown in medium 199.

The importance of folic acid compounds for development of the agent finds additional confirmation in the inhibiting effect of aminopterin and its competitive reversal by folinic acid observed in the present experiments. These findings are consistent with the reports of Colon(23) and Colon and Moulder(24) on the presence of folic acid synthesizing enzyme in elementary bodies of psittacosis virus and on the synthesis of folinic acid in tissues infected by the agent. The function of folic acid compounds is obviously connected with the intensive nucleic acid metabolism of these agents, which in the course of their development synthesize large amounts, first of RNA and then of DNA containing material (11,25). The sequence of these syntheses forms a fascinating object for further biochemical studies.

It can also be inferred from these results that the agent of trachoma, in its behavior to antibiotics and sulfa compounds, closely resembles other members of the psittacosis lymphogranuloma group(26).

Summary. FL cell cultures infected with the T'ang strain (TE55) of trachoma have been used to evaluate the effect of a number of antibiotics and a sulfa drug. Complete inhibition was observed by doses of 0.1 μg/ml of tetra- and oxytetracycline. Chloramphenicol, erythromycin and chlortetracycline were effective in 10-100 times higher doses. Penicillin prevented the appearance of infective particles in doses of 0.1 unit/ml. Lower doses of the antibiotics showed a partial inhibiting effect. Both penicillin and tetracycline also inhibited the agent when added in the later stages of its developmental cycle. The sulfa drug sulfanilamido-3phenyl-2pyrazole prevented the appearance of infective particles in doses of 100 μg/ml. Its inhibitory effect was limited to the early developmental stages of the agent, and could be reversed by PABA, folinic and folic acid. Aminopterin, like the sulfa drug, inhibited the agent at an early stage of development. No inhibition was observed when large doses of streptomycin, bacitracin and neomycin were employed.

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A Regulated Incubator Controlling CO_2 Concentration, Humidity and Temperature for Use in Animal Cell Culture.* (27707)

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Clonal plating of mammalian cells requires regulation of temperature, humidity and CO_2 concentration within narrow limits. Although small experiments can be carried out in sealed desiccators or plastic bags containing appropriate gas mixtures, operation on a larger scale requires an incubator which can be opened and closed repeatedly without fluctuations in temperature or gaseous composition.

An incubator which is continuously flushed with a humidified carbon dioxide-air mixture has been described elsewhere(1), and has been successfully employed for several years. However, such incubators depend on a constant rate of delivery of clean, compressed air, which is frequently not available. Moreover, even with the highest rates of gas flow which are practicable, recovery of the steady state conditions after opening of the incubator door is slow enough to cause serious difficulties in a heavily used incubator.

For these reasons, a new incubator was designed, in which the CO_2 concentration is continuously monitored, and pure CO_2 is injected automatically as needed to maintain the desired level. Water vapor is continuously supplied by a humidifier located inside the incubator. The arrangement is illustrated in Fig. 1 and 2.

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Principle of operation. Carbon dioxide concentration is monitored by measuring the pH of a standardized sodium bicarbonate solution through which gas from the incubator is continuously passed. The pH meter operates a switching circuit which causes the injection of additional CO_2 whenever the concentration falls below a predetermined level.

The following equipment is needed for construction of the carbon dioxide control apparatus shown in Fig. 2. (The apparatus as shown is designed for use with a double-door, water jacketed incubator with 2 thermometer ports in its top, and with sufficient space between its left and right side shelves for the blower duct system. Application to other incubators will require some alteration of the design. The numbers refer to the location of items in Fig. 2.)

1. "Standard" tank of liquid CO_2 (delivers gaseous CO_2), National Cylinder Gas Medical Grade, or equivalent.

2. Two stage automatic regulator. Matheson No. 8, with No. 320 connection.

3. Low pressure shutoff valve (supplied as part of item 2).

4. Solenoid valve. Skinner V52DA2100 or equivalent. Normally closed. ¼″ NPT connections.

5. Combination needle valve and flow gauge. Hoke style 993, with meter 2142. 0.2 - 1.5 l.p.m., calibrated for CO_2.